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Short Communication

# Conformationally restrained ceramide analogues: effects of lipophilic modifications on the antiproliferative activity

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**Abstract**

Conformationally restrained analogues of ceramide containing thiouracil or uracil moieties in their polar head, substituted with an ethyl group in their 6-positions, proved to inhibit cell proliferation and induce apoptosis. A series of new thiouracil and uracil analogues of ceramide possessing several 6-alkyl- or 6-arylalkyl-substituents, were synthesized and tested as inhibitors of cell proliferation. The lipophilic substituents introduced in the 6-position were pure alkyls (*n*-propyl, *n*-butyl, *i*-butyl, *neo*-pentyl), or aryl-alkyls (2-phenylethyl). Although a significant antiproliferative activity was maintained in most compounds synthesized, none of them showed any improvement with respect to their 6-ethyl-substituted counterparts.


*Keywords:* Apoptosis; Ceramide analogues; Thiouracil; Uracil; Lipophilicity

## 1. Introduction

Ceramide, a lipidic second messenger involved in the sphingomyelin cycle, plays a crucial role in the processes of induction of apoptosis and inhibition of cell proliferation [1]. Its metabolism involves a hydrolysis step operated by the enzyme ceramidase to produce sphingosine, which is then phosphorylated by a specific kinase to sphingosine-1-phosphate, a metabolite which, unlike ceramide, stimulates cell growth and proliferation (Fig. 1). The dynamic balance between levels of sphingolipid metabolites, ceramide and sphingosine-1-phosphate, is an important factor that determines whether a cell survives and proliferates or undergoes an apoptotic process. Another metabolic pathway that produces ceramide is a de novo synthesis operated by a ceramide synthase, in response to stress signals [2]. An increased production of intracellular ceramide, both by sphingomyelin hydrolysis and by de novo synthesis, is stimulated by stress factors (including ionizing radiation, cytokines, etc.) occurring outside the cell membrane [3,4], which eventually cause the apoptotic death of the cell. As a consequence, the suppression of the production of growth inhibitory lipids, such as ceramide, may lead to uncontrolled cell proliferation, which eventually develops cancer.

It has actually been verified that there is a close correlation between cancer cell proliferation and low levels of intracellular ceramide [5]. Therefore, either inhibition of the ceramide metabolism (inhibition of ceramidase) or the use of ceramide mimics would open promising perspectives in cancer therapy, by restoring the growth inhibitory effect of endogenous ceramide and regulating the abnormal growth in tumor cells. Ceramide itself cannot be used since it is too lipophilic to cross the cell membrane. Appropriate ceramide mimics should be metabolically stable, able to permeate through the membrane and to reach reasonable intracellular

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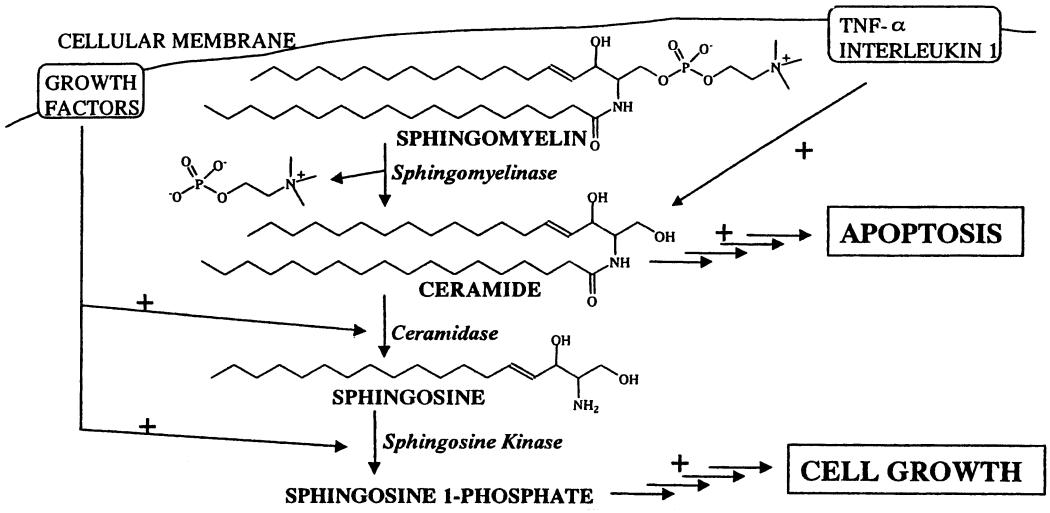
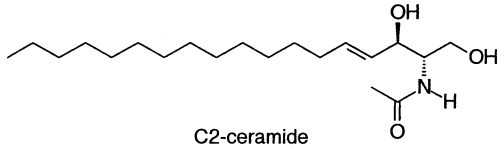



Fig. 1. Schematic representation of the sphingomyelin cycle and effect of extracellular factors on ceramide generation cell proliferation and death.

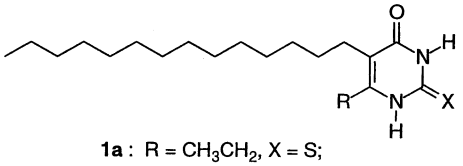
concentrations. Some early examples of ceramide mimics, which showed promising results were C2- and C6-ceramide [5], in which the long side chain at the amidic portion is replaced by an acetyl or a hexanoyl group, respectively. Other attempts to produce ceramide analogues concerned the replacement of the alkenyl chain with a styryl [6] or an allylic fluoride [7], or the introduction of a sugar moiety, to form α-galactosylceramides [8].

We have recently synthesized new conformationally restricted analogues of C2-ceramides, compounds **1a** and **2a** [9], which, respectively, possess a thiouracil (X = S) or a uracil (X = O) ring in the place of the polar head of C2-ceramide. Moreover, they are substituted in the 6-position of the polar ring with an ethyl side chain (R = $CH_3CH_2$), closely resembling the structural motif of C2-ceramide. Both compounds (**1a** and **2a**) showed excellent antiproliferative properties when tested on the human T-cell acute leukemia cells CCRF-CEM and they proved to be practically devoid of any toxicity when administered (up to 200 mg/kg) to animal models [9].

On the basis of these results, we have developed a series of new analogues of compounds **1a** and **2a**, bearing bulkier and more lipophilic substituents in the place of the simple 6-ethyl group. We, therefore, synthesized thiouracil and uracil derivatives containing an *n*-propyl (**1b**, **2b**), an *n*-butyl (**1c**, **2c**), an *i*-butyl (**1d**, **2d**), a *neo*-pentyl (**1e**, **2e**), or a 2-phenylethyl (**1f**, **2f**) group in their 6- positions.




C2-ceramide




**1a** : R = $CH_3CH_2$, X = S;
**2a** : R = $CH_3CH_2$, X = O;

**1b** : R = $CH_3CH_2CH_2$, X = S;
**2b** : R = $CH_3CH_2CH_2$, X = O;

**1c** : R = $CH_3CH_2CH_2CH_2$, X = S;
**2c** : R = $CH_3CH_2CH_2CH_2$, X = O;

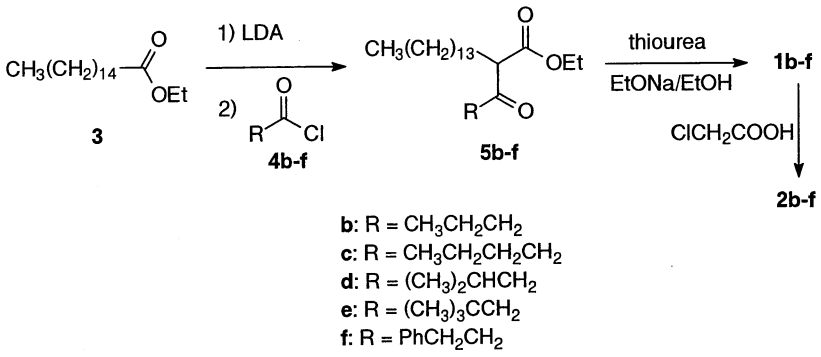
**1d** : R = $(CH_3)_2CHCH_2$, X = S;
**2d** : R = $(CH_3)_2CHCH_2$, X = O;

**1e** : R = $(CH_3)_3CCH_2$, X = S;
**2e** : R = $(CH_3)_3CCH_2$, X = O;

**1f** : R = $PhCH_2CH_2$, X = S;
**2f** : R = $PhCH_2CH_2$, X = O.

## 2. Chemistry

Compounds **1b**–**f** and **2b**–**f** were synthesized as shown in Scheme 1. Ethyl palmitate (**3**) was treated with lithium diisopropylamide (LDA) in THF to generate the corresponding lithium enolate, which was then reacted with the appropriate acid chloride (**4b**–**f**) to produce the related β-ketoester **5b**–**f**, which was not purified, but was used as a crude intermediate for the next step. Subsequent condensation of **5b**–**f** with thiourea in the presence of sodium ethoxide in refluxing absolute ethanol afforded thiouracil derivatives **1b**–**f** [10]. The thioracil ring was then converted into a uracil ring by exchanging the sulfur with an oxygen atom provided by reaction of compounds **1b**–**f** with a refluxing 10% aqueous solution of chloroacetic acid, affording uracil derivatives **2b**–**f** [11,12].




Scheme 1.

## 3. Results and discussion

The antiproliferative activities of compounds **1b**–**f** and **2b**–**f** were assessed in the human T-cell acute leukemia cells CCRF-CEM, and were compared with the data previously obtained with compounds **1a** and **2a** [9], using C2-ceramide as the reference compound. The results are reported in Table 1.

All the new thiouracil (**1b**–**f**) and uracil (**2b**–**f**) derivatives, which differ from **1a** and **2a** because they contain higher homologues of their 6-ethyl substituent, generally proved to possess appreciable antiproliferative activities, although they are lower than that of **1a** and **2a**. Among them, **1d** and **2d**, containing isobutyl substituents in their 6-positions, proved to be the most active of this new series, with $IC_{50}$s of 13.2 and 8.7 μM, respectively, a value which is still better than the $IC_{50}$ obtained with C2-ceramide (31.6 μM). In general the differences in the activities obtained with the new compounds are not remarkable and, therefore, it is not possible to highlight rational effects of the different character of the alkyl- and arylalkyl-side chains introduced in the 6-position of reference compounds **1a** and **2a**. Furthermore, also in this series, there are no significant differences between the activities of thiouracil (**1b**–**f**) and uracil (**2b**–**f**) derivatives.

Table 1
Effects on cell growth ($IC_{50}$, inhibitory concentration at the 50% effect level) by compounds **1a**–**f**, **2a**–**f** and C2-ceramide

| Comp. | $IC_{50}$ (μM) [a] |
|---|---|
| **1a** | 1.7±0.2 [b] |
| **2a** | 7.9±0.9 [b] |
| **1b** | 50.1±7.2 |
| **2b** | 35.2±5.0 |
| **1c** | 31.6±5.8 |
| **2c** | 50.1±4.3 |
| **1d** | 13.2±2.3 |
| **2d** | 8.7±1.2 |
| **1e** | 15.1±1.9 |
| **2e** | 29.1±3.5 |
| **1f** | 20.7±2.6 |
| **2f** | 15.6±1.5 |
| C2-ceramide | 31.6±4.6 [b] |

[a] $P < 0.05$. ANOVA followed by the Tukey–Kramer post test, versus C2-ceramide.
[b] Ref. [9].

The decrease in the activity observed with these new derivatives (**1b**–**f** and **2b**–**f**) with respect to the previously reported ones (**1a** and **2a**) [9] might be due to their increased lipophilicity, which could be responsible for a lower efficiency in the delivery process of these molecules inside the tumor cells, where they produce their antiproliferative effect.

These results tend to indicate that in general substituents of a simple aliphatic and aromatic nature in the 6-position of thiouracil (type **1**) and uracil (type **2**) derivatives do not significantly disturb their antiproliferative activities, although small ethyl groups (**1a** and **2a**) seem to be preferred. As no improvements were obtained by increasing the lipophilic character of the 6-substituents, it would be wise in the near future to synthesize new compounds of both types (**1** and **2**) containing in this same position polar moieties, in order to evaluate the effects of a lowered lipophilicity in this region on the cell-proliferation inhibitory properties of these kinds of molecules.

## 4. Experimental

### 4.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. $^1$H NMR spectra of all compounds were obtained with a Varian Gemini-200 instrument operating at 200 MHz; the data are reported as follows: chemical shift (in ppm) from the $Me_4Si$ line as the external standard, multiplicity (br, broad; s, singlet; d, doublet; t, triplet; m, multiplet). Mass spectra were recorded on a VG 70-250S mass spectrometer. Analytical TLCs were carried out on 0.25 mm layer silica gel plates containing a fluorescent indicator; spots were detected under UV light (254 nm). Column chromatography was performed using 230–400 mesh

silica gel (Macherey–Nagel Silica Gel 60 Art. no. 815381). Sodium sulfate was always used as the drying agent. Evaporations were performed in vacuo (rotating evaporator). Synthesis and characterization data of compounds **1a** and **2a** have already been published [9]. C2-ceramide was purchased from Sigma, St. Louis, MO.

*4.1.1. Synthesis of thiouracil derivatives **1b–f***

A solution obtained by dissolving 2.0 g of ethyl palmitate (7.0 mmol) in 5 ml of anhydrous THF was added drop by drop, at a temperature of 0 °C under an argon gas atmosphere, to 4.2 ml of a 2 M solution of LDA in anhydrous THF. After 30 min of stirring at 0 °C, the reaction mixture was added to a solution containing 8.5 mmol of the appropriate acyl chloride (**4b–f**) in 10 ml of anhydrous THF. The resulting mixture was stirred at room temperature (r.t.) for 12 h, then added to a saturated solution of $NH_4Cl$. The organic solvent was removed under a vacuum and the remaining aqueous phase was extracted with diethyl ether. The combined organic phase was washed with a saturated aqueous solution of NaCl, dried over anhydrous $Na_2SO_4$ and then concentrated under a vacuum to provide a crude residue composed almost exclusively of β-ketoester (**5b–f**), which was used in the next step without further purification. The crude residue was dissolved in 40 ml of absolute ethanol and then treated with 8.02 g of thiourea (52.8 mmol) and 14.4 g of sodium ethoxide (106 mmol). The resulting mixture was stirred at 90 °C for 1 h, after which time it was cooled to r.t. and filtered. The filtrate was evaporated under a vacuum; the residue thus obtained was dissolved in a 10:1 mixture of water and THF. The resulting solution was cooled to 0 °C and acidified to pH 2 with concentrated HCl; the precipitate that developed due to acidification was collected by suction filtration and washed with small quantities of acetone, providing a crude residue which was purified by silica gel column chromatography using an *n*-hexane–EtOAc mixture as the eluant, to afford pure thiouracils **1b–f**.

Compound **1b**: 644 mg, 1.76 mmol, 25% yield (two steps). M.p. 128–130 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.87 (t, 3H, $J = 6.4$ Hz), 1.03 (t, 3H, $J = 7.2$ Hz), 1.25 (br, 26H), 2.35 (t, 2H, $J = 7.4$ Hz), 2.43 (t, 2H, $J = 7.5$ Hz), 9.73 (br, 2H, $D_2O$ exchangeable). MS (EI, 70 eV): $m/z$ 366 ($M^+$).

Compound **1c**: 642 mg, 1.69 mmol, 24% yield (two steps). M.p. 124–126 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.87 (t, 3H, $J = 6.8$ Hz), 0.96 (t, 3H, $J = 7.2$ Hz), 1.10–1.48 (m, 28H), 2.34 (t, 2H, $J = 7.5$ Hz), 2.46 (t, 2H, $J = 7.6$ Hz), 10.12 (br, 2H, $D_2O$ exchangeable). MS (EI, 70 eV): $m/z$ 380 ($M^+$).

Compound **1d**: 748 mg, 1.97 mmol, 28% yield (two steps). M.p. 102–104 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.87 (t, 3H, $J = 6.4$ Hz), 1.00 (d, 6H, $J = 6.6$ Hz), 1.12–1.39 (m, 24H), 1.93–2.03 (m, 1H), 2.31–2.38 (m, 4H), 10.20 (br, 1H, $D_2O$ exchangeable), 10.45 (br, 1H, $D_2O$ exchangeable). MS (EI, 70 eV): $m/z$ 380 ($M^+$).

Compound **1e**: 859 mg, 2.18 mmol, 31% yield (two steps). M.p. 58–60 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.88 (t, 3H, $J = 6.5$ Hz), 1.06 (s, 9H), 1.16–1.36 (m, 24H), 2.31–2.46 (m, 4H), 8.92 (br, 1H, $D_2O$ exchangeable), 9.37 (br, 1H, $D_2O$ exchangeable). MS (EI, 70 eV): $m/z$ 394 ($M^+$).

Compound **1f**: 903 mg, 2.11 mmol, 30% yield (two steps). M.p. 100–102 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.88 (t, 3H, $J = 6.3$ Hz), 1.11–1.40 (m, 24H), 2.27–2.96 (m, 6H), 7.18–7.34 (m, 5H), 9.65 (br, 1H, $D_2O$ exchangeable), 9.72 (br, 1H, $D_2O$ exchangeable). MS (EI, 70 eV): $m/z$ 428 ($M^+$).

*4.1.2. Synthesis of uracil derivatives **2b–f***

The appropriate thiouracil derivative **1b–f** (0.45 mmol) was treated with a 10% aqueous solution of chloroacetic acid (11 ml) and the mixture was refluxed for 16 h. The resulting precipitate was then collected by suction filtration, washed with absolute ethanol and diethylether. The crude product so obtained was purified by silica gel column chromatography using an *n*-hexane–EtOAc mixture as the eluant, to afford pure uracils **2b–f**.

Compound **2b**: 52 mg, 0.15 mmol, 33% yield. M.p. 132–134 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.87 (t, 3H, $J = 6.4$ Hz), 1.01 (t, 3H, $J = 7.3$ Hz), 1.25 (br, 26H), 2.32 (t, 2H, $J = 7.5$ Hz), 2.42 (t, 2H, $J = 7.7$ Hz), 8.81 (br, 1H, $D_2O$ exchangeable), 9.74 (br, 1H, $D_2O$ exchangeable). MS (EI, 70 eV): $m/z$ 350 ($M^+$).

Compound **2c**: 57 mg, 0.16 mmol, 35% yield. M.p. 116–120 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.87 (t, 3H, $J = 6.6$ Hz), 0.96 (t, 3H, $J = 7.1$ Hz), 1.16–1.46 (m, 28H), 2.32 (t, 2H, $J = 7.8$ Hz), 2.43 (t, 2H, $J = 7.6$ Hz), 8.72 (br, 1H, $D_2O$ exchangeable), 9.53 (br, 1H, $D_2O$ exchangeable). MS (EI, 70 eV): $m/z$ 364 ($M^+$).

Compound **2d**: 53 mg, 0.14 mmol, 32% yield. M.p. 105–108 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.88 (t, 3H, $J = 6.4$ Hz), 0.99 (d, 6H, $J = 6.6$ Hz), 1.14–1.40 (m, 24H), 1.93–2.03 (m, 1H), 2.31–2.36 (m, 4H), 9.08 (br, 1H, $D_2O$ exchangeable), 9.91 (br, 1H, $D_2O$ exchangeable). MS (EI, 70 eV): $m/z$ 364 ($M^+$).

Compound **2e**: 63 mg, 0.17 mmol, 37% yield. M.p. 98–100 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.88 (t, 3H, $J = 6.3$ Hz), 1.00 (s, 9H), 1.15–1.40 (m, 24H), 2.33–2.45 (m, 4H), 8.37 (br, 1H, $D_2O$ exchangeable), 8.85 (br, 1H, $D_2O$ exchangeable). MS (EI, 70 eV): $m/z$ 378 ($M^+$).

Compound **2f**: 72 mg, 0.18 mmol, 39% yield. M.p. 118–120 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.88 (t, 3H, $J = 6.3$ Hz), 1.13–1.37 (m, 24H), 2.23–2.92 (m, 6H), 7.21–7.32 (m, 5H), 8.79 (br, 1H, $D_2O$ exchangeable), 9.93 (br, 1H, $D_2O$ exchangeable). MS (EI, 70 eV): $m/z$ 412 ($M^+$).

### 4.2. Assay of cell proliferation [9]

The human T-cell leukemia cell line CCRF-CEM (American Type Culture Collection, Manassas, VA), was used to test the inhibitory effect on cell proliferation of compounds **1b**–**f** and **2b**–**f**. C2-ceramide was used as the reference compound. CCRF-CEM cells were maintained in RPMI 1640 medium supplemented with fetal calf serum (10%), glutamine (2 mM), penicillin (50 IU/ml) and streptomicin (50 μg/ml) (HyClone, Cramlington, UK), in an atmosphere of 5% $CO_2$ and 95% air at 37 °C. Cell proliferation was assessed by the CellTiter 96 (Promega, Madison, MA) based on the cellular metabolism of the tetrazolium compound XTT. Compounds **1b**–**f**, **2b**–**f**, and C2-ceramide were dissolved in sterile dimethyl sulfoxide (DMSO) at 10 mM and stored at −20 °C; drugs were further diluted in sterile culture medium immediately before their use. CCRF-CEM cells were seeded at 5000 cells per 200 μl of culture medium containing compounds **1b**–**f**, **2b**–**f**, and C2-ceramide at 0.01–50 μM into each well of a 96-well microtiter plate, and incubated at 37 °C with 5% $CO_2$ for 48 h. Control cultures received the vehicle only (DMSO or sterile water). At the end of drug exposure, 50 μl of medium containing 50 μg of XTT and 0.38 μg of phenazine sulfate were added; cells were incubated for an additional 3–4 h and the absorbance measured at 450 nm with a microplate reader (MicroReader, Bio-Rad, Manassas, VA). Inhibition of proliferation was assessed as the percentage reduction of UV absorbance of treated cells versus control cultures and the 50% inhibitory concentration of cell growth ($IC_{50}$) was calculated by non-linear least-squares curve fitting.

### 4.3. Data analysis

Experimental data are expressed as mean values ± standard deviation and statistical comparisons are obtained by analysis of variance (ANOVA) followed by the Tukey–Kramer post test to compare pairs of group means.

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